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The Monomeric Glutamyl-tRNA Synthetase of *Escherichia coli*

PURIFICATION AND RELATION BETWEEN ITS STRUCTURAL AND CATALYTIC PROPERTIES*

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The glutamyl-tRNA synthetase has been purified to homogeneity from *Escherichia coli* with a yield of about 50%. It is a monomer with a molecular weight of 56,000 and has the same kinetic properties as those of the α chain of the dimeric αβ-glutamyl-tRNA synthetase described previously (Lapointe, J., and Söll, D. (1972) *J. Biol. Chem.* 247, 4966–4974). It is the smallest aminoacyl-tRNA synthetase purified from *E. coli* and contains no important sequence repetition. It is also the only monomeric aminoacyl-tRNA synthetase reported so far to contain no major sequence duplication. Considering its structural and mechanistic similarities with the glutaminyl- and the arginyl-tRNA synthetases of *E. coli*, we propose the existence of a relation between the true monomeric character of the glutamyl-tRNA synthetase (as opposed to monomers with sequence duplications) and its requirement for tRNA in the activation of glutamate.

A single sulfhydryl group of the native enzyme reacts with 5,5′-dithiobis(2-nitrobenzoic acid) causing no loss of enzymatic activity, whereas four such groups per enzyme react in the presence of 4 M guanidine HCl.

According to the theory of the co-evolution of the genetic code and of the amino acids biosynthetic pathways (1, 2), glutamate is one of the seven amino acids present in the "paleokaryotes." In this context, the glutamyl-tRNA synthetase is probably one of the "oldest" aminoacyl-tRNA synthetases. This model is supported by the unusual properties of this enzyme (3) and the absence of a glutaminyl-tRNA synthetase in *Bacillus subtilis* (3, 4). A better understanding of its structure and properties might help to unify our view of the structure of the aminoacyl-tRNA synthetases (5–7) and of the evolution of their structural genes.

The purification of a dimeric form (αβ) of this enzyme from *Escherichia coli* has been reported previously (8). Following a separation of these two "subunits" by a mild procedure (isoelectric focusing), only the α polypeptide (M_r = 56,000) can catalyze the formation of Glu-tRNA, while the β polypeptide (M_r = 46,000) increases the affinity of α for glutamate and ATP and its stability (9). In view of the weakness of the interaction between α and β (9), we now consider the glutamyl-tRNA synthetase as a monomeric enzyme (α) which can interact and is sometimes co-purified with the β polypeptide.

This monomeric enzyme constitutes, with the glutaminyl-tRNA synthetase and the arginyl-tRNA synthetase, a subgroup of aminoacyl-tRNA synthetases sharing the following structural and catalytic properties. They require their cognate tRNA to catalyze the incorporation of [^{32}P]PP_i into ATP, and they are monomeric enzymes of similar molecular weights, respectively, 56,000, 68,000, and 64,000 (8, 10, 11). The glutamyl-tRNA synthetase appears to a have a strategic position in this family since glutamate is a metabolic precursor to both glutamine and arginine. It is also the smallest monomeric aminoacyl-tRNA synthetase of this subgroup and of all the aminoacyl-tRNA synthetases studied up to now in *E. coli*.

We describe here a new technique for the purification of this monomeric enzyme with a yield of about 50%. We have studied some of its properties and compared them to those of the αβ enzyme. We suggest a model for the evolution of the structural genes of the aminoacyl-tRNA synthetases specific for glutamate, glutamine, and arginine and present a correlation between the fact that these three synthetases are monomers, and their requirement for tRNA to catalyze the ATP-PP_i exchange. The catalytic mechanism of this monomeric enzyme will be described elsewhere.[1]

EXPERIMENTAL PROCEDURES

Materials—*E. coli* MRE-600 was grown in minimal medium (12) and harvested during the exponential growth phase by centrifugation. The wet cells were frozen with liquid nitrogen and stored at −20°C. For some purifications, we started from 100,000 × *g* supernatants of MRE-600 extracts, kindly provided by Dr. A. H. Wahba (13).

Unfractionated tRNA from *E. coli* B was purchased from Schwarz/Mann. It contains 4% $tRNA^{Glu}$. Phenylmethylsulfonyl fluoride (PMSF),[2] 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *p*-hydroxymercuribenzoate (*p*-HMB), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), bovine serum albumin, and ATP were obtained from Sigma, and Omnifluor, uniformly labeled L-[^{14}C]glutamate, and sodium [^{32}P]pyrophosphate from New England Nuclear. Microgranular DEAE-cellulose (DE52) was bought from Whatman, hydroxylapatite (HA) from Bio-Rad, the various chemicals for making polyacrylamide gels and 2-mercaptoethanol from Eastman Chemicals, polyethylene glycol 6000 from J. T. Baker, and dextran T-500 from Pharmacia.

Methods—Protein concentrations were determined according to Lowry (14), or for pure enzyme solutions, by their optical density at 280 nm using the relation: $\epsilon_{280\ nm}$ = 0.87 (*cf.* "Results").

Aminoacylation Reaction—The formation of Glu-tRNA was followed in 0.1-ml reaction mixtures containing 50 mM sodium Hepes, pH 7.2, 2 mM ATP, 3 mg of tRNA/ml, and the indicated amounts of enzyme. When necessary, the enzyme was diluted in 10 mM sodium

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[1] D. Kern, and J. Lapointe, manuscript in preparation.

[2] The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); *p*-HMB, *p*-hydroxymercuribenzoate; Hepes, 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid); TEMED, *N,N,N′,N′*-tetramethylethylenediamine; SDS, sodium dodecyl sulfate; albumin, bovine serum albumin; *p*-CMB, *p*-chloromercuribenzoate; PEG, polyethylene glycol; and PAGE, polyacrylamide gel electrophoresis.

Hepes, pH 7.2, 20 mM 2-mercaptoethanol, and 1 mg of albumin/ml. After various incubation times at 37°C, an aliquot was transferred on a disc of Whatman No. 3MM filter paper, washed successively during 15 min in each of three 5% trichloroacetic acid solutions at 0°C, two 95% ethanol solutions, and finally in diethyl ether. The dried filters were placed in a solution of 4 g of Omnifluor/liter of toluene and counted for ^{14}C in a Beckman LS-355 scintillation counter.

Incorporation of [^{32}P]PP$_i$ into ATP—The reaction mixture contained, unless otherwise mentioned, 100 mM sodium Hepes, pH 7.2, 2 mM ATP, 16 mM $MgCl_2$, 6 mM L-glutamate, 3 mM [^{32}P]PP$_i$ (about 2000 cpm/nmol), 1.5 to 3.0 mg of tRNA/ml, and various amounts of enzyme. After various incubation times at 37°C, the ATP present in the reaction mixture was specifically adsorbed on acid-washed Norit as described previously (8), rinsed with 15 ml of 0.1 M NaPP$_i$ on Whatman Fiberglas filter discs CF/c or on Whatman No. 3MM filter paper discs, dried, and counted for ^{32}P in a solution of 4 g of Omnifluor/liter of toluene.

Kinetic Measurements—All substrates, except one whose concentration was variable, were present at such concentrations that their specific binding sites on the glutamyl-tRNA synthetase were saturated (2 mM ATP, 0.3 mM L-glutamate, 1 μM tRNA^{Glu} in unfractionated tRNA from *E. coli*). The other components of the reaction mixtures for the aminoacylation reaction or for the incorporation of [^{32}P]PP$_i$ into ATP were as described above. Reactions were started by adding the enzyme solution, previously equilibrated at 37°C. The amounts of [^{14}C]Glu-tRNA or of [^{32}P]ATP formed during at least three different incubation times were used to determine the initial velocity of the reaction. The kinetic data were analyzed according to Lineweaver and Burk (15).

Concentration of Cellular Fractions—Three techniques were used to concentrate various cellular fractions: dialysis against a buffer solution containing 30% (w/v) polyethylene glycol 6000, dialysis under vacuum across a membrane (Schleicher and Schuell), or adsorption on a small DEAE-cellulose (DE52) column followed by elution in a small volume by a salt solution.

Preparative Electrophoresis—It was conducted according to Davis (16) in a Canalco gel electrophoresis apparatus. The lower gel (40 ml) was formed at room temperature from a solution containing 7.5% acrylamide, 0.3% bisacrylamide, 0.365 M Tris-HCl, pH 8.9, 15 μl of *N,N,N′,N′*-tetramethylethylenediamine (TEMED), and 3 mg of ammonium persulfate. After its polymerization, unreacted persulfate ions were removed by a 30-min electrophoresis. Then, 2 ml of solution containing 2.5% acrylamide, 0.65% bisacrylamide, 0.06 M Tris-HCl, pH 6.8, 1 μl of TEMED, 1 mg of ammonium persulfate, and 0.01 mg of riboflavin were poured over the first gel and polymerized by UV irradiation. The top of the upper gel and the bottom of the lower gel were connected respectively to the cathode and the anode by a buffer (pH 8.3) containing 6 g of Tris (base) and 28.8 g of glycine/liter. Sometimes, the upper gel was omitted and the protein solution previously dialyzed against 0.06 M Tris-HCl, pH 6.8, and 50% glycerol was poured on the gel. During the electrophoresis, the external surface of the column was kept at 0° to 4°C with a stream of water.

Molecular Weight Determination—In the absence of a denaturing agent, the molecular weight was measured either by sedimentation on sucrose gradient under conditions described previously (17) or by electrophoresis on polyacrylamide gels of various concentrations (18). In the presence of a denaturing agent, we followed the protocol of Weber and Osborn (19).

Sulfhydryl Group Titration—We used the method described by Ellman (20). A solution containing a few milligrams of enzyme was first dialyzed two times during 8 h against 5 liters of 10 mM Tris-HCl, pH 7.4, to remove all traces of 2-mercaptoethanol. The titration of the sulfhydryl groups with DTNB in this buffer was followed by measuring the $A_{412\ nm}$ in a 0.5-cm path length quartz cell with a Cary 15 recording spectrophotometer.

Amino Acids Analysis—Samples were thoroughly dialyzed against dilute acetic acid (0.5% v/v), freeze-dried, and hydrolyzed under nitrogen in 6 N HCl (containing 0.5% (v/v) thiodiglycol and a crystal of phenol) at 110°C for 25 h. Analyses were performed with a Beckman Unichrom Analyzer. Cysteine and methionine were determined as cysteic acid and methionine sulfone, respectively, after hydrolysis of the performic-oxidized enzyme according to Moore (21). Tryptophan was estimated following the method of Liu and Chang (22).

Carboxymethylation and Tryptic Digestion—Enzyme samples were carboxymethylated, using a technique derived from that of Crestfield *et al.* (23). Samples (3.5 mg/ml) were reduced with 10 mM dithioerythritol for 1 h at room temperature and thoroughly dialyzed under nitrogen against 0.2 M Tris-HCl, pH 8.5, 0.1 mM EDTA, and 0.1 mM diisopropyl fluorophosphate. Solid recrystallized urea was used to yield a final concentration of 8 M and the resulting solution was incubated for 1 h at 37°C. [^{14}C]Iodoacetate (300 μg, ~4 μCi/μmol) were added and the labeling was carried out under nitrogen at room temperature for about 30 min (15-fold excess of the reagent over the thiol groups). A second addition of 20 μl of nonradioactive 1 M iodoacetate solution was made and the reaction was continued for another 30 min. During the whole incubation with iodoacetate, the vial was wrapped in aluminum foil to minimize iodine formation. The excess of reagent was destroyed by addition of 1% (v/v) 2-mercaptoethanol and the solution was exhaustively dialyzed against a 1% (w/v) solution of ammonium bicarbonate.

That solution was freeze-dried and redissolved in 0.2 ml of 1% ammonium bicarbonate. Trypsin was added (1:100, w/w) and the mixture was incubated at 37°C for 4 h, freeze-dried, dissolved in the minimum amount (40 μl) of 10% acetic acid, and fingerprinted.

Fingerprinting Techniques—The above solution was subjected to thin layer electrophoresis and chromatography on cellulose plates. Electrophoresis was run in the first dimension in a CAMAG apparatus at pH 4.4 (pyridine:acetic acid:acetone:water, 40:80:300:1580). The plates were dried and run in the second dimension in the following solvent: butan-1-ol:acetic acid:water:pyridine (15:3:12:10). With these combined techniques, about 100 μg of peptides in 10 μl could be fingerprinted and analyzed. Peptides were detected by ninhydrin staining (spray with 0.3% ninhydrin in ethanol, containing 3% collidine and 10% acetic acid). Radioactive peptides were detected by autoradiography of the map. Arginine-containing peptides were revealed by their fluorescence after the map was sprayed with a solution of 0.01% phenanthrenequinone and 5% NaOH in anhydrous ethanol (24). Tryptophan-containing peptides were specifically labeled with the Ehrlich reagent (1%, w/v, *p*-dimethylaminobenzaldehyde in 10 ml of 12 N HCl, dissolved in 90 ml of acetone).

RESULTS

Purification of the Glutamyl-tRNA Synthetase

All the operations were performed between 0° and 4°C. All the buffers contained 10% (v/v) glycerol, 20 mM 2-mercaptoethanol, and 0.1 mM PMSF as protective agents against proteases. In the buffer used for cell lysis, 10 mM PMSF was present. The centrifugations were made in a GSA rotor in a Sorvall RC2-B.

Step 1: Cell Lysis—Wet cells (1 kg) were suspended in 2 liters of 10 mM potassium phosphate, pH 8.0, and broken by sonication during 10 min in a Raytheon sonic oscillator (model DF101), by fractions of 75 ml. The lysate was centrifuged at 8000 rpm during 30 min to remove cell debris and intact cells, yielding 2350 ml of supernatant.

Step 2: Partition in a Polyethylene Glycol-Dextran Two-Phase System—Potassium phosphate, pH 8.0 (125 ml, 1 M), was added to the supernatant for reasons described previously (8). Then, concentrated solutions of PEG-6000 and dextran T-500 were added to reach the final concentrations of 7 and 1.5%, respectively, in the supernatant. This suspension was mixed during 2 h, and the two phases were separated by centrifugation at 5000 rpm during 20 min. The PEG-rich top phase contains most of the glutamyl-tRNA synthetase activity.

Step 3: Chromatography on DEAE-cellulose—The top phase (3 liters) was diluted by addition of 2 liters of 10% glycerol, 20 mM 2-mercaptoethanol, to reduce the ionic strength. Half of the solution (2.5 liters) was adsorbed on a column (7 × 30 cm) of DEAE-cellulose (type DE52), which was then washed with 1 liter of 10 mM potassium phosphate, pH 7.5. The (macro)molecules left on the column were then eluted at about 400 ml/h with a linear salt and pH gradient of 6 liters (20 mM potassium phosphate, pH 7.5, to 250 mM potassium phosphate, pH 6.5) (Fig. 1*A*). The glutamyl-tRNA synthetase activity was eluted near the end of this gradient, and was already separated from most other aminoacyl-tRNA synthetases.[1] The most active fractions were pooled and di-

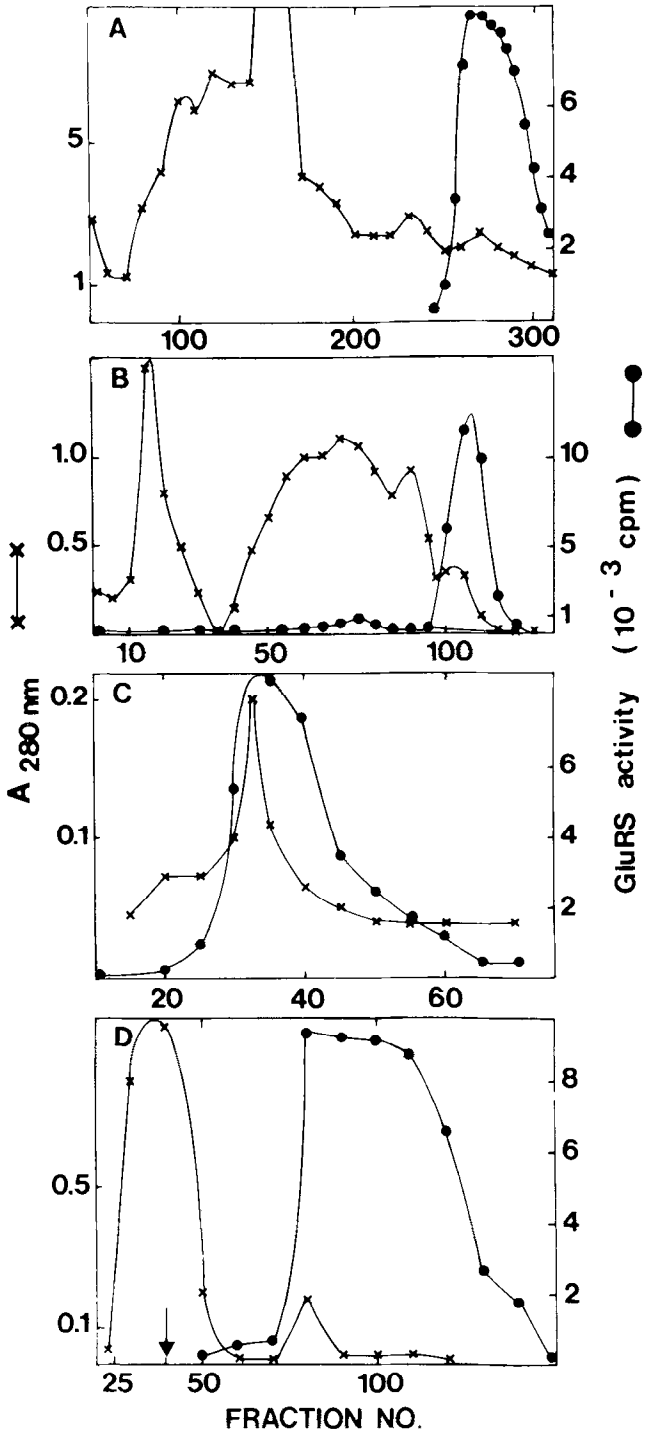

FIG. 1. Various steps of the purification of the glutamyl-tRNA synthetase (*GluRS*). *A*, chromatography on DEAE-cellulose. Fractions of 20 ml were collected. *B*, chromatography on hydroxylapatite (16-ml fractions). *C*, electrophoresis on polyacrylamide gel (8-ml fractions). *D*, chromatography on phosphocellulose (20-ml fractions). The *arrow* shows which fraction was collected when 0.1 M KCl was added to the elution buffer. For the enzyme assays, dilutions (*A*, 13; *B*, 21; *C*, 6; *D*, 170 times) of various fractions were made in the aminoacylation mixture. Following an incubation of about 10 min at 37°C, the amounts of [^{14}C]Glu-tRNA present in 50-μl samples of the mixtures were determined as described under "Experimental Procedures."

alyzed against 10 mM potassium phosphate, pH 6.8 (Fraction DEAE). The same purification step was conducted on the other half (2.5 liters) of the diluted top phase.

Step 4: Chromatography on Hydroxylapatite—Fraction DEAE obtained from the chromatography of the 5 liters of diluted top phase was adsorbed on a hydroxylapatite column (6 × 12 cm) equilibrated against 10 mM potassium phosphate, pH 6.8. The column was washed at 300 ml/h with 200 ml of the same buffer, then with 2 liters of a linear gradient from 20 to 200 mM potassium phosphate, pH 6.8. Two peaks of glutamyl-tRNA synthetase activity were eluted (Fig. 1*B*); the first, representing only a small percentage of the total activity was eluted at a conductivity of 3.6 mmho (at 4°C), whereas the second and major peak was eluted at 5.7 mmho. Only the most active fractions of this major peak were pooled (Fraction HA) and used in the following purification steps.

Step 5a: Preparative Polyacrylamide Gel Electrophoresis: Last Purification Step—Fraction HA was concentrated 2- to 3-fold by dialysis against 30% polyethylene glycol and then against 0.01 M Tris, 0.077 M glycine (pH 8.3), 20 mM 2-mercaptoethanol, 50% glycerol. About 100 mg of proteins present in 8 ml of this concentrated Fraction HA were mixed with 0.1 ml of a saturated solution of bromphenol blue and layered on the top of a column (12 cm × 3.4 cm^2) of polyacrylamide gel whose preparation is described under "Experimental Procedures." A constant current of 40 mA was passed through the gel, whose electric resistance gradually reached a constant value of about 10,000 ohms. The bottom surface of the gel was continuously washed with a 80-ml/h stream of 0.01 M Tris, 0.077 M glycine (pH 8.3), 10% glycerol, and 20 mM 2-mercaptoethanol, which was collected in 8-ml fractions. Following the elution of bromphenol blue (4 h of electrophoresis), the glutamyl-tRNA synthetase was the first protein eluted (Fig. 1*C*), as was observed for the $\alpha\beta$ enzyme (8). The active fractions were pooled and concentrated (Fraction PAGE).

Step 5b: Chromatography on Phosphocellulose: Alternative Last Purification Step—Because no more than 100 mg of proteins from Fraction HA could be purified to homogeneity by electrophoresis on the polyacrylamide gel column described above, we replaced this step in certain cases by a chromatography of Fraction HA dialyzed against 10 mM potassium phosphate, pH 7.0, on a column (3.5 × 35 cm) of phosphocellulose (Whatman P-11) equilibrated against the same buffer. After adsorption of the protein sample, the column was washed at a rate of 100 ml/h with 750 ml of this buffer, then with the same buffer containing 0.1 M KCl. Under these conditions, the glutamyl-tRNA synthetase was retarded compared to all the other proteins present in the Fraction HA (Fig. 1*D*). The fractions containing this enzymatic activity were pooled and concentrated (Fraction Phosphocellulose).

Purity of the Glutamyl-tRNA Synthetase Obtained after Steps 5a and 5b—The analysis of the Fraction PAGE by analytical polyacrylamide gel electrophoresis in the absence or presence of a denaturing agent revealed, respectively, the presence of one protein band (Fig. 2*A*) and of one polypeptide chain (Fig. 2*B*), indicating that this fraction contains only the pure enzyme. A similar analysis of Fraction Phosphocellulose shows that the glutamyl-tRNA synthetase represents more than 90% of its protein content (Fig. 2*C*).

Yield of these Purifications—The results of two purification procedures, using as the last step an electrophoresis on polyacrylamide gel and a chromatography on phosphocellulose, respectively, are summarized in Table I, A and B. For the first purification (Table IA), the cell extract was obtained by sonication of cells grown as described under "Experimental Procedures." The final step (electrophoresis) had to be performed three times because of the low capacity of our column. For the second purification procedure (Table IB), the starting material was a 100,000 × *g* supernatant (*cf.* "Experimental Procedures"). The specific activity of the glutamyl-tRNA synthetase obtained with the second procedure is slightly superior to that of the Fraction PAGE obtained with the first

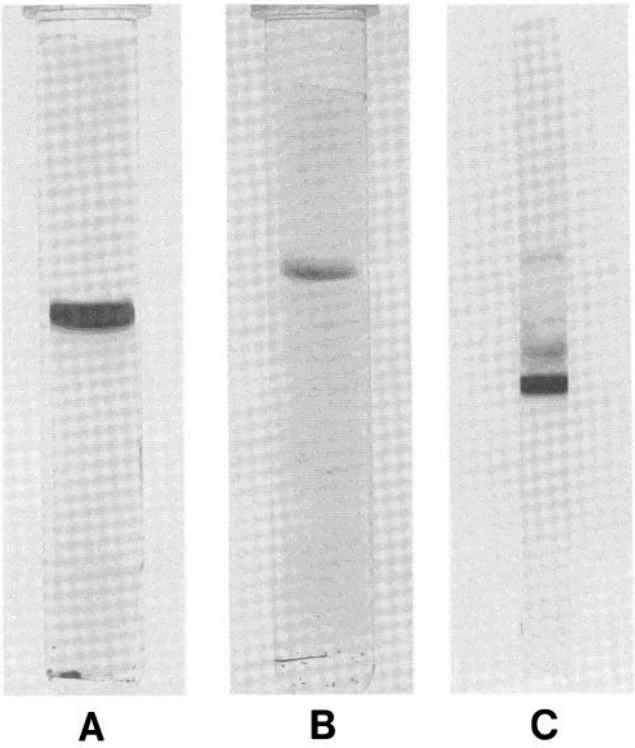

FIG. 2. Analytical electrophoresis on polyacrylamide gel. A sample of the enzyme Fraction PAGE was analyzed by electrophoresis in the absence (*A*) and in the presence (*B*) of reducing and denaturing agents. *Gel C* shows the analysis of a sample of reduced and denatured Fraction Phosphocellulose.

TABLE I

Purification of the glutamyl-tRNA synthetase

(A) First procedure, starting from 1 kg of *E. coli* MRE-600

Step	Total protein	Total units[a]	Specific activity	Recovery
	mg		*units/mg*	%
1. Cell extract	31,090	42,060	1.35	100
2. Liquid polymer extract	18,060	40,000	2.20	95
3. DEAE-cellulose	1,630	37,490	23.0	89
4. Hydroxylapatite	210	28,980	138.	69
5a. Electrophoresis	25	19,750	790.	47

(B) Second procedure, starting from the high speed supernatant from 2 kg of *E. coli* MRE-600

Step	Total protein	Total units[a]	Specific activity	Recovery
	mg		*units/mg*	%
1. 105,000 × *g* supernatant	43,000	96,100	2.23	100
2. DEAE-cellulose	4,640	88,800	18.1	92
3. Hydroxylapatite	620	77,900	125	87
4. Phosphocellulose	57	61,600	1071	64

[a] One unit of enzyme catalyzes the formation of 1 nmol of Glu-tRNA/min at 37°C.

procedure, suggesting that a small proportion of the enzyme molecules is inactivated during the preparative electrophoresis. The molar extinction coefficient of the Fraction Phosphocellulose at 280 nm (ϵ_{280}) is 0.87.

Turnover Number of the Pure Glutamyl-tRNA Synthetase—The rate of formation of Glu-tRNA is optimal at pH 8.6, whereas the $tRNA^{Glu}$-dependent incorporation of [^{32}P]PP_i into ATP is optimal at pH 6.2.[1] One molecule of pure enzyme catalyzes the formation of 3.4 molecules of Glu-tRNA/s at pH 8.6 at 37°C.

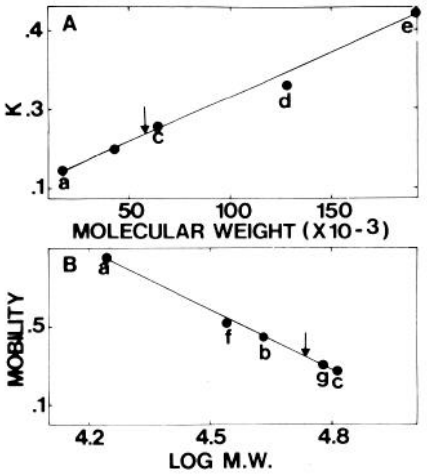

FIG. 3. Molecular weight of the native and of the denatured glutamyl-tRNA synthetase. The following proteins were used as molecular weight markers: *a*, myoglobin (17,200); *b*, ovalbumin (43,000); *c*, bovine serum albumin monomer (68,000); *d*, albumin dimer; *e*, albumin trimer; *f*, pepsin (35,000); *g*, catalase (60,000). The *arrows* indicate the mobility of the glutamyl-tRNA synthetase. *A*, estimation of the molecular weight of the native enzyme by disc electrophoresis on polyacrylamide gels of various concentrations, 5, 6, 7.5, 9, and 10%. About 15 μg of Fraction PAGE and of (M_r) markers were used. The value *K* for each protein is calculated from its mobility in various concentrations of polyacrylamide gels by the equation, mobility (relative to that of the bromphenol blue) = $K \times$ gel concentration. The correlation reported by Hedrick and Smith (18) was used to estimate the molecular weight of the native enzyme. *B*, determination of the molecular weight of the reduced and denatured enzyme. Following their incubation for 10 min at 90°C in 1% SDS, 1% 2-mercaptoethanol, 0.01 M sodium phosphate, pH 7.2, about 15 μg of Fraction PAGE and of (M_r) markers were analyzed by electrophoresis on a 10% polyacrylamide gel, and the molecular weight of the denatured enzyme was determined (19).

TABLE II

Amino acid composition of the monomeric glutamyl-tRNA synthetase of E. coli

Amino acid	Approximate number of residues per 56,000
Alanine	44
Arginine	31
Aspartic acid + asparagine	56
Cysteine[a]	5
Glutamic acid + glutamine	70
Glycine	34
Histidine	13
Isoleucine	25
Leucine	39
Lysine	26
Methionine[a]	15
Phenylalanine	15
Proline	21
Serine	23
Threonine	25
Tryptophan	5
Tyrosine	16
Valine	31
Total	494

[a] Determined as cysteic acid and methionine sulfone, respectively.

Some Structural Properties of the Glutamyl-tRNA Synthetase

Molecular Weight and Monomeric Structure—In the absence of a denaturing agent, the molecular weight of the native enzyme has been determined by sedimentation on sucrose gradient in presence of either catalase (M_r = 240,000), alcohol dehydrogenase (M_r = 150,000), or hemoglobin (M_r = 64,500).

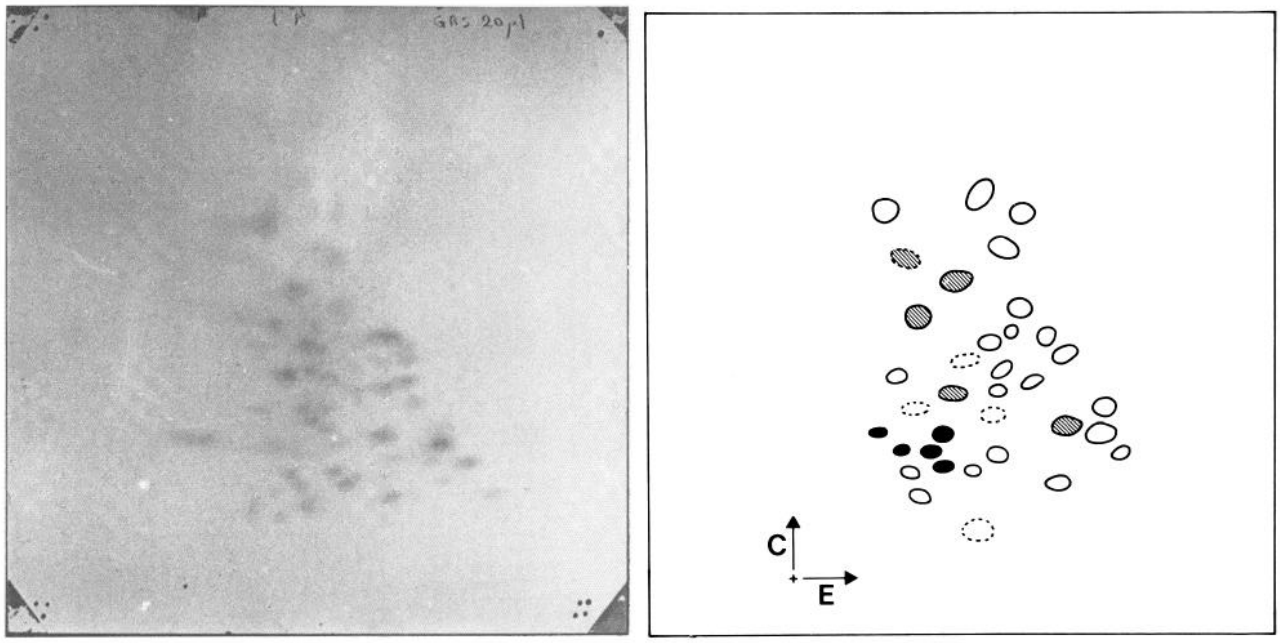

FIG. 4. Map of the tryptic peptides of 10 nmol of reduced and [^{14}C]carboxymethylated Fraction PAGE. After application of the aliquot on a cellulose thin layer plate at the origin (+), the peptides were separated first by electrophoresis at pH 4.4 (*E*) during 1½ h at 400 V, then by ascending chromatography (*C*). The *photograph* represents the map after ninhydrin staining. The *drawing* shows the position of the arginine-containing peptides (after phenanthrenequinone staining). Among these, the cysteine-containing peptides correspond to the *black spots* (autoradiography of the plate) and those containing tryptophan to *dashed circles* (Ehrlich staining).

The values obtained are, respectively, 53,100, 71,100, and 60,800. By electrophoresis of the native enzyme in polyacrylamide gels of various concentrations in the presence of M_r markers, we obtained a value of about 58,000 (Fig. 3*A*).

The reduced and denatured enzyme migrates, during electrophoresis in the presence of SDS (*cf.* "Experimental Procedures"), as a single polypeptide chain (Fig. 2*B*) of M_r = 56,000 (Fig. 3*B*). These results indicate that the native enzyme is a monomer.

Amino Acid Composition and Tryptic Map—The amino acid composition of the glutamyl-tRNA synthetase is presented in Table II. A two-dimensional analysis of a tryptic digest of the pure enzyme previously labeled with [^{14}C]iodoacetate reveals the presence of about 55 peptides (Fig. 4). About 30 peptides contain arginine, 5 contain tryptophan, and 5 react with [^{14}C]iodoacetate. The amino acid analysis of these 5 ^{14}C-labeled peptides shows the presence of one carboxymethylcysteine per peptide, indicating that only cysteines have reacted with [^{14}C]iodoacetate. These results are in agreement with the amino acid composition: 31 arginines, 26 lysines, 5 tryptophans, and 5 cysteines or half-cystines per enzyme molecule (Table II).

Titration of the Sulfhydryl Groups of the Glutamyl-tRNA Synthetase with DTNB and p-CMB and their Influence on the Enzymatic Activities—When a solution of enzyme was dialyzed twice for 12 h successively against 1000 times its volume of a neutral buffer containing no sulfhydryl group protector, about 50% of its aminoacylation and of its ATP-[^{32}P]PP$_i$ exchange activities was lost. The initial activity could be completely recovered by addition of 20 mM 2-mercaptoethanol. On the other hand, dialysis against the same buffer containing no O_2 (removed by a stream of N_2) did not inactivate it. The titration of sulfhydryl groups was conducted in the absence and in the presence of a denaturing agent.

In the absence of a denaturing agent, the reaction of DTNB with the native enzyme (followed by measuring the change of absorbance at 412 nm) is completed after 30 min, when 0.96 sulfhydryl group/enzyme molecule has reacted with DTNB (Fig. 5). The kinetics of this reaction is biphasic. Half of the

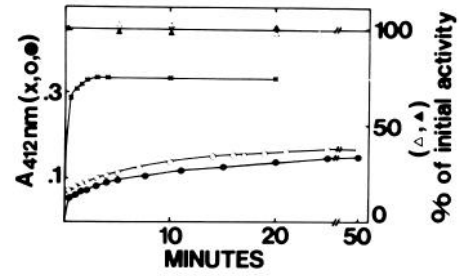

FIG. 5. Titration of the sulfhydryl groups of the glutamyl-tRNA synthetase with DTNB, and parallel measurement of its aminoacylation activity. These experiments were conducted at 25°C, in a 0.5-cm pathlength cell as described under "Experimental Procedures." For the titration in the presence of 100 μM DTNB (ϵ = 13,600), the concentration of Fraction PAGE was 24 μM in the absence of a denaturing agent (●) and in the presence of 5 M urea (○), and it was 12 μM in the presence of 4 M guanidine HCl (×). For the determination of the glutamyl-tRNA synthetase activity, 10-μl samples of enzyme (25 μM) preincubated in the presence of 0.1 mM (△) or 1 mM (▲) DTNB were diluted in 1 ml of 0.01 M Tris-HCl, pH 7.4. This dilution was assayed for aminoacylation activity as described under "Experimental Procedures."

sulfhydryl groups able to react under these conditions do so during the 1st min while the other half has completely reacted only about 30 min later, suggesting the existence of at least two populations (*e.g.* populations with different conformations) of enzyme molecules differing in the reactivity of their sulfhydryl groups with DTNB. Even a 50-fold excess of DTNB (1 mM) over the enzyme concentration (0.02 mM) did not diminish its aminoacylation activity (Fig. 5) nor its ATP-[^{32}P]PP$_i$ exchange activity (results not shown) over an incubation period of 30 min. On the other hand, *p*-HMB strongly inhibits both of these catalytic activities in parallel (Fig. 6). In the presence of a stoichiometric concentration of this reagent, 50% of the activity is lost after 2 min, and 80% after 30 min. A 3-fold excess of *p*-HMB causes the loss of 85% of the glutamyl-tRNA synthetase activity within 2 min.

In the presence of 5 M urea, the reaction of the glutamyl-

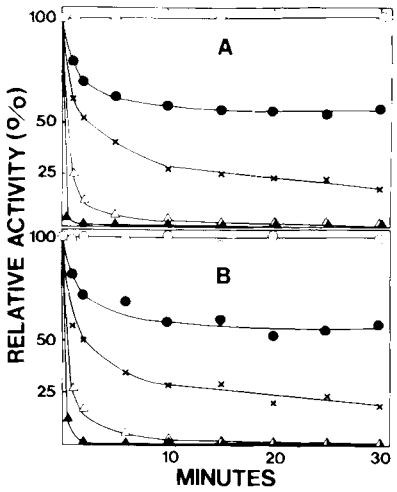

FIG. 6. Influence of *p*-hydroxymercuribenzoate (*p*-HMB) on the aminoacylation (*A*) and on the ATP-[^{32}P]PP$_i$ isotope exchange (*B*) reactions catalyzed by the glutamyl-tRNA synthetase. *A*, Fraction PAGE (0.59 μM) was incubated in 120 μl in the absence (○) or in the presence of 0.5 μM (●), 1 μM (×), 3 μM (△), or 5 μM (▲) *p*-HMB. After various incubation times, 5-μl samples were mixed with 45 μl of 0.01 M Tris-HCl, pH 7.5, at 0°C. The residual aminoacylation activity was measured by incubating 10 μl of this dilution with 90 μl of aminoacylation assay mixture at 37°C during 5 min, and determining the amount of [^{14}C]Glu-tRNA in 50-μl samples. We verified that under these conditions, the initial velocity of the aminoacylation reaction was measured. *B*, the conditions of reaction with *p*-HMB and dilution were identical with those described above. The rate of the ATP-[^{32}P]PP$_i$ isotope exchange reaction was measured at pH 6.2 as described under "Experimental Procedures."

TABLE III

Comparison of the K_m of the monomeric glutamyl-tRNA synthetase and of the αβ enzyme (9) for their substrates, measured at pH 7.2

	Glutamate	ATP	$tRNA^{Glu}$
	μM	μM	μM
Monomeric (α)	100	180	0.08
αβ (*cf.* Ref. 9)	5	40	0.30
α derived from αβ (*cf.* Ref. 9)	86	250	0.12

tRNA synthetase with DTNB is over after 30 min, when 2.08 sulfhydryl groups/enzyme have reacted. Here again, the reaction has a faster initial phase, 0.9 sulfhydryl group/enzyme reacting during the 1st min (Fig. 5). In the presence of 4 M guanidine HCl, 4.1 sulfhydryl groups/enzyme react with DTNB within 2 min (Fig. 5).

Comparison of the Kinetic Properties of the Monomeric and of the αβ-Glutamyl-tRNA Synthetases

The K_m values of the monomeric enzyme for its substrates were measured at the same pH (7.2) used for the determination of those of the αβ enzyme and of the α polypeptide derived from it (9). As shown in Table III, the K_m of the monomeric enzyme whose purification is described here is very similar to those of α obtained by isoelectric focusing of αβ (8). These two monomeric glutamyl-tRNA synthetases also have a lower affinity for glutamate and ATP than that of the αβ form.

DISCUSSION

The Dimeric Glutamyl-tRNA Synthetase—A dimeric structure of the type αβ has been proposed (8) for the glutamyl-tRNA synthetase of *E. coli* on the basis of the following observations. Following five purification steps, the enzymatic activity co-migrates with one protein band during gel electrophoresis in the absence of a denaturing agent. An analysis of this protein by gel electrophoresis in the presence of SDS shows the presence of approximately equimolar amounts of two polypeptide chains of M_r = 56,000 (α) and 46,000 (β). Finally, the polypeptide chains α and β were separated by isoelectric focusing; α, but not β, had glutamyl-tRNA synthetase activity. The β chain increased the thermal stability of α and its affinity for glutamate and ATP in the aminoacylation reaction (9).

The interactions observed between α and β were relatively weak. Indeed, they could be separated by isoelectric focusing, and the αβ enzyme sediments as a globular protein of M_r = 60,000 on sucrose gradient (8). This interaction is thus much weaker than that observed between the subunits of other aminoacyl-tRNA synthetases, and is compatible with the regulatory function suggested for β by its *in vitro* properties (9). Consequently, the properties and the interaction of α and β are such that we now consider α as a monomeric glutamyl-tRNA synthetase and β as a polypeptide chain which can interact with it. The physiological role of the β protein is still unknown, but its co-purification with α is not purely accidental since it does influence its activity (9) and was found in several independent purifications. Moreover, the glutamyl-tRNA synthetase of *B. subtilis* is a monomer of M_r = 65,000, and is also weakly associated with a protein of $M_r \approx$ 46,000 (3). The possibility that the β protein is a product of the partial proteolysis of α is extremely weak. Indeed, its calculated amino acid composition is very different from that of α (Ref. 8, and Table II). Moreover, partially proteolyzed forms of several aminoacyl-tRNA synthetases generally retain some catalytic activity and do not co-purify with the intact form. For instance, the partially proteolyzed form of the valyl-tRNA synthetase from yeast is active and is eluted from hydroxylapatite before the native enzyme (25). In this context, the very minor peak of glutamyl-tRNA synthetase activity eluted from the hydroxylapatite column before the major one (Fig. 1*C*) is likely to be due to a partially proteolyzed enzyme. This possibility is strengthened by the fact that this first peak of activity eluted from hydroxylapatite is much smaller when the serine proteases inhibitor PMSF is present during the purification, than in the absence of protease inhibitor (results not shown).

It is conceivable that the association between α and β may be due to relatively labile disulfide bond(s), since both polypeptides migrate together during electrophoresis on polyacrylamide gel in the absence of a reducing agent, whereas their interaction was not detected during a sedimentation of the αβ enzyme on a H_2O:D_2O density gradient in the presence of 0.01 M 2-mercaptoethanol (8).[3]

New Purification Procedure of the Monomeric Glutamyl-tRNA Synthetase—The approach described here for the purification of this enzyme has two major differences from the one leading to the αβ form (8). First, we used a different linear salt and pH gradient to elute the enzyme from the DEAE-cellulose column. Secondly, none of the buffers used for this purification contained $MgCl_2$. The enzyme obtained after four purification steps is a single polypeptide chain of M_r = 56,000. In the absence of a denaturing agent, it migrates on a gel (Fig. 3) or sediments on a sucrose gradient as a globular protein of M_r = 55,000 to 60,000, indicating a structure of the type α. This monomeric enzyme corresponds, by its structural (M_r = 56,000), catalytic, and kinetic properties (Ref. 9, and Table III) to the α subunit of the αβ complex described previously (8, 9). While we were working on this project, Willick and Kay (26) reported the purification of the "catalytically active subunit" of the glutamyl-tRNA synthetase using a modification of our initial procedure (8).

[3] S. Yang and D. Söll, personal communication.

Structural Features of the Monomeric Glutamyl-tRNA Synthetase—The number of tryptic peptides (about 55) obtained from the monomeric enzyme which contains 57 basic amino acids (26 lysines and 31 arginines) is as expected from a polypeptide chain containing no significant amount of repetitive amino acid sequences. A more detailed analysis of the map showed that about 30 peptides contain arginine, 5 contain tryptophan, and 5 contain a cysteine (or half-cystine). Considering that one enzyme molecule contains 31 arginines, 5 tryptophans, and 5 cysteines (or half-cystines), we conclude that the monomeric glutamyl-tRNA synthetase is the largest aminoacyl-tRNA synthetase reported so far to contain no sequence duplication.

In the absence of a denaturing agent, one DTNB reacts with each enzyme molecule without inactivating it (Fig. 5), indicating that this thiol is not involved in the catalytic activity of the enzyme. The DTNB is apparently not detached from the enzyme in the aminoacylation mixture since, under the same conditions, very low *p*-HMB concentrations inactivate it (Fig. 6). One cysteine or half-cystine appears to be well hidden in the enzyme structure since only four out of five found by amino acid analysis can react with DTNB in the presence of 5 M guanidine HCl (Fig. 5).

The Glutamyl-tRNA Synthetase and the Group of Small Monomeric Aminoacyl-tRNA Synthetases in E. coli—The glutamyl-tRNA synthetase is the smallest monomeric aminoacyl-tRNA synthetase of *E. coli*. It is noticeable that the two other aminoacyl-tRNA synthetases of *E. coli*, which share the exceptional catalytic property of requiring their cognate tRNAs to catalyze the incorporation of [^{32}P]PP_i into ATP, are also monomeric enzymes whose molecular weights are in the same range. The arginyl-tRNA synthetase has a molecular weight of about 64,000 (11) and the glutaminyl-tRNA synthetase of 69,300 (10). In *B. subtilis*, no glutaminyl-tRNA synthetase has been detected, and one glutamyl-tRNA synthetase (M_r = 65,000) catalyzes the acylation of glutamate on both $tRNA^{Glu}$ and $tRNA^{Gln}$ (3).

The theory of the co-evolution of the genetic code and of the amino acids biosynthetic pathways (1, 2) proposes that today's codons for glutamine and arginine were originally coding for glutamate in paleokaryotes, and were attributed to glutamine and arginine at the time of the development of the enzymatic pathways for their biosynthesis from glutamate. In this context, and considering the primitive structural and catalytic properties of the glutamyl-tRNA synthetase and its similarities with the glutaminyl- and the arginyl-tRNA synthetases, we will test the possibility that the structural gene(s) for the glutaminyl-tRNA synthetase (and maybe also for the arginyl-tRNA synthetase) evolved by mutations of a gene coding for a primitive glutamyl-tRNA synthetase.

Monomerism and "Activation" by tRNA—Considering the structural and catalytic similarities between the aminoacyl-tRNA synthetases specific for glutamate, glutamine, and arginine in *E. coli*, we expect that no major sequence duplication will be found in the latter two enzymes. Preliminary results with the arginyl-tRNA synthetase from yeast agree with this prediction. A complete confirmation of this expectation will establish that these three enzymes are the only monomeric aminoacyl-tRNA synthetases containing no major sequence duplication. This implies that they would be the only aminoacyl-tRNA synthetases containing only one copy of a fundamental sequence of the order of 500 amino acids. Indeed, all other aminoacyl-tRNA synthetases whose sequences have been studied in *E. coli* are either monomers with an internal repetition of such a fundamental sequence, or dimers, or tetramers (5–7, 27). It is interesting to correlate this (partly predicted) fundamental monomerism of these three synthetases with their requirements for their cognate tRNAs in the catalysis of the incorporation of [^{32}P]PP_i into ATP. Both properties are indicative of primitive structures.

Consequently, *we propose the existence of a relation between the real monomerism of the glutamyl-tRNA synthetase, and its property of requiring its cognate tRNA to activate glutamate.* We predict that the same relation exists for the arginyl- and the glutaminyl-tRNA synthetases.

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